Impact of transdermal flunixin administration on serum prostaglandin E\textsubscript{2} and cortisol concentrations in piglets following castration

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https://doi.org/10.2460/ajvr.21.12.0201

OBJECTIVE
To assess the effects of transdermal flunixin administration on serum prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) and cortisol concentrations in piglets undergoing castration.

ANIMALS
104 litters with at least 4 male piglets/litter.

PROCEDURES
Litters were randomly assigned to 1 of 4 treatments: transdermal flunixin (3.33 mg/kg) administration followed by surgical castration (CF; n = 28), transdermal flunixin administration followed by sham castration (SF; n = 26), application of physiologic saline solution followed by sham castration (S; n = 26), and application of physiologic saline solution followed by surgical castration (C; n = 24). Blood samples were collected 24 hours before and 1, 4, and 25 hours after castration or sham castration.

RESULTS
Serum PGE\textsubscript{2} concentrations for piglets in the C and CF groups did not differ at any time. Piglets in the S group tended to have higher serum PGE\textsubscript{2} concentrations 1 hour after sham castration compared with piglets in the SF group. One hour after the procedure, piglets that underwent castration had higher serum cortisol concentrations than did piglets that underwent sham castration. Piglets in the CF group had higher serum cortisol concentrations than did piglets in the SF group 4 hours after the procedure, but serum cortisol concentrations did not differ between the C and S groups.

CLINICAL RELEVANCE
Further studies are needed to explore dosing regimens, including effective doses and administration frequencies, and the pharmacokinetics of flunixin following transdermal administration in piglets undergoing castration.
In addition to mitigating inflammation, NSAIDs are also effective in reducing pain. Physiologic parameters are important when assessing pain in animals, and previous studies have identified serum cortisol concentration as a relevant parameter to evaluate stress and pain in pigs. Previous authors have used serum cortisol concentration to determine drug efficacy in relieving pain in piglets following castration.

In the United States, flunixin is commonly used on swine farms to control pyrexia associated with swine respiratory disease and has documented success in mitigating pain associated with sow lameness. Most recently, the US FDA has approved the use of flunixin as a topical pour-on for use in cattle to mitigate pain associated with foot rot. Topical administration of products is a unique option for pain mitigation on swine farms, because it provides an attractive option that producers can implement on a large scale. However, to the authors’ knowledge, no studies to date have evaluated the anti-inflammatory and analgesic effects of transdermal flunixin administration in pigs undergoing castration. The objective of the study reported here was to assess the effects of transdermal flunixin administration on inflammation and stress, as determined by measuring serum PGE2 and cortisol concentrations, in piglets undergoing castration.

Materials and Methods

The study protocol was approved by the Institutional Animal Care and Use Committee of North Carolina State University (protocol No. 20-113-01). Animals were cared for and handled in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching. The study was conducted from January to March 2021 at a commercial swine breeding facility located in the southeastern United States.

Animals, housing, and management

A total of 1,445 Large White X Duroc cross male piglets from 109 litters were used in the study. Sows (first through sixth lactation) and piglets (mean of 11 piglets/litter) were housed in fully slatted and mechanically ventilated farrowing rooms. In each room, sows were housed in individual farrowing crates (2.5 X 0.7 m) with additional space for the piglets (2.5 X 1.3 m) surrounding the crates. Room lights were on between 6:00 am and 4:30 pm, and sows were provided ad libitum access to water and feed throughout the study. Piglets had access to the sow and a water nipple at all times.

Study design and treatments

Litters were included in the study if the litter consisted of a minimum of 4 male piglets (cross-fostering was permitted prior to study enrollment), piglets were ≤ 8 days old, the piglets had intact tails, both testicles had descended in all piglets, and piglets weighed > 0.5 kg. Litters were excluded from the study if the sow or piglets had any clinical signs of disease, if the sow or piglets had been treated with any antimicrobial, or if the sow received additional treatments from the farm personnel. Litters enrolled in the study did not undergo any additional processing procedures (eg, tail docking, teeth clipping, iron supplementation, or antimicrobial administration) during the time of the study.

Male piglets in enrolled litters were ear tagged (Global piglet ear tags; Allflex Livestock Intelligence) for identification purposes, and litters were randomly assigned to 1 of 4 treatments: transdermal flunixin (Banamine transdermal; 3.33 mg/kg) administration followed by surgical castration (CF), transdermal flunixin administration followed by sham castration (SF), application of physiologic saline solution followed by sham castration (S), and application of physiologic saline solution followed by surgical castration (C). Randomization was done with the RAND function in Excel (Microsoft Corp). Treatments were applied at the litter level to avoid any potential within-litter drug carryover effects.

Drug administration

Twenty-four hours before castration was performed, flunixin (3.33 mg/kg) or physiologic saline solution (equivalent volume) was applied topically by a single individual next to the tail head along the dorsal midline with a disposable syringe (BD disposable syringes; Fisher Scientific). Treatment volumes ranged from 0.1 to 0.3 mL/piglet. Corn starch and a pink nontoxic dye were added to the saline solution to minimize observer bias throughout the study by mimicking the consistency and color of the flunixin solution. To ensure the modified saline solution did not cause local or systemic reactions, a small subset of piglets in which the modified solution had been applied to the skin were observed for any reactions.

Castration procedure

Castration was performed in a routine manner by 2 farm personnel previously trained to perform the procedure and who had > 7 years of experience castrating piglets. Piglets assigned to undergo surgical castration were held in an inverted position, and the testicles were pushed into the scrotum. A vertical incision was made over each testicle with a cutting pliers instrument. The testicle was extruded through the incision and grasped with the thumb and forefinger of the person performing the procedure, and the testicle was removed with the spermatic cord and surrounding tunics. The incisions were sprayed with iodine and left open to heal, and piglets were returned to the farrowing crate.

To account for handling, piglets assigned to the sham castration groups were handled in a manner similar to that for castrated piglets, which included picking up each individual piglet from the crate and holding it in an inverted position against the body of the person performing the procedure. This person then applied pressure to the scrotal area with a thumb and finger but did not incise the skin. Iodine was then applied to the scrotal area, and the piglet was returned to the farrowing crate.
Blood sample collection

Blood samples were collected from a randomly selected subset of 94 piglets 24 hours before and 1 and 25 hours after castration or sham castration for determination of serum PGE2 concentrations. In addition, blood samples were collected from a randomly selected subset of 438 piglets 24 hours before and 1, 4, and 25 hours after castration or sham castration for determination of serum cortisol concentrations.

Blood samples were collected by puncture of the orbital sinus with a disposable 20-gauge, 1-inch needle (Vacutainer; Becton, Dickinson and Company) with the rubber sheath removed as previously described.25 All blood tubes were maintained in a cooler and centrifuged at 2,000 X g for 15 minutes at 4° C within 6 hours after sample collection and serum was harvested. Serum aliquots were stored at −80° C, and assays were performed 2 months later. For determination of serum PGE2 and cortisol concentrations, 375 and 20 μL, respectively, were used.

Determination of serum PGE2 concentration

Prostaglandin E2 concentrations were measured with a commercial enzyme-linked immunosorbent assay (catalog No. 514531; Cayman Chemical) as previously described.26 Briefly, samples were purified by adding a volume of ice-cold acetone equal to 4 times the volume of the serum sample. Then, samples were incubated at −20° C for 30 minutes and centrifuged at 3,000 X g for 5 minutes. The supernatant was transferred to a 13 X 100-mm glass tube, evaporated with a concentrator (CentriVap; Labconco), and reconstituted to the initial serum volume with kit buffer. An aliquot of the reconstituted sample was derivatized with adjusted kit components; the manufacturer's protocol was then followed. Samples were analyzed in duplicate. Absorbance was measured at 405 nm following 60 minutes of development (SpectraMax i3; Molecular Devices). Mean PGE2 concentration of a sample used to evaluate repeatability among plates was 12.3 pg/mL. Intra- and interassay coefficients of variation were 19% and 13.2%, respectively.

Determination of serum cortisol concentration

Serum cortisol concentrations were quantified using a commercial enzyme-linked immunosorbent kit (DetectX cortisol EIA kit; Arbor Assays). Upper and lower detection limits of the assay were 50 and 3,200 pg/mL, respectively. Samples were diluted 1:100 with assay buffer and analyzed according to kit directions. All samples were assayed in duplicate. In total, 40 assay kits were used. Mean ± SD intra-assay coefficient of variation for duplicate samples was 6.7 ± 7.5%. Mean interassay coefficient of variation for 2 quality-control samples was 10.0 ± 0.1%.

Statistical analysis

Multivariable mixed-effects linear regression models were separately built at the piglet level for the 2 outcomes of interest: PGE2 concentration and cortisol concentration. Treatment and time were included as main effects. For model building, the first step was assessing linearity of the association between all continuous variables and the outcomes of interest with linear smooth plots. When the linearity assumption was not met, the variable was dichotomized at the median. Second, univariable mixed-effect models were built for each of other captured variables, which included piglet age (continuous, in days), lactation of the sows (continuous, in number), parity of the sows (categorical [primiparous or multiparous]), weekly mortality rate (continuous, in percentage), and body weight of the piglets (continuous, in kg). In addition, cortisol concentration (continuous, in ng/mL) was considered as a fixed effect for the PGE2 model. Variables with a P value ≤ .20 in the univariable analysis were eligible to be tested for inclusion in the final multivariable model. Third, the Spearman correlation method was used to check for collinearity between all independent predictors, with a cutoff of 0.80.

Lastly, final multivariable mixed-effect linear models were constructed with a backwards stepwise approach, starting with all predictors with values of P ≤ .20. To investigate the combined effects of treatment and time on the outcomes, the 3-way interaction between drug, procedure, and time relative was included in all models. In all models, 2 random effects were included: litter, to account for clustering of piglets within litters, and piglet, to account for repeated measurements on individual animals. During construction of the final models, confounders were assessed by removing the variables individually and assessing other variable’s coefficients for a change of ≥ 20%. If this was the case, variables were retained in the models. Adjusted linear predictions for contrasts between castration procedure and drug with 95% CIs were estimated with the delta method. Statistical significance was declared at P ≤ .05. All analyses were performed with standard software (Stata, version 15; StataCorp).

Results

One-hundred nine litters were enrolled in the study, but 5 litters were removed because the sows received additional treatment by farm personnel. Therefore, 104 litters were used in the analyses. Mean age of piglets in these 104 litters was 3 days (SD, 0.9 days; range, 2 to 8 days) and mean body weight was 2.1 kg (SD, 0.5 kg; range, 1.03 to 3.40 kg) at the time of enrollment. Of the 104 litters, 28 were assigned to the CF group, 26 were assigned to the SF group, 26 were assigned to the S group, and 24 were assigned to the C group.

Effect of drug and procedure on serum PGE2 concentration

The final multivariate model for serum PGE2 concentration retained weekly mortality rate (P = .005) and body weight of the piglets (P = .002) as predictors. Serum PGE2 concentration of piglets in the C and CF groups did not differ significantly (P ≥ .26) at any time (Figure 1). Similarly, serum PGE2 concentration of piglets in the SF group did not differ from
concentration for piglets in the S group at baseline ($P = .7$) or 25 hours after sham castrations ($P = .3$). Piglets in the S group tended to have ($P = .09$) higher serum PGE$_2$ concentrations 1 hour after sham castration than did piglets in the SF group.

**Effect of drug and procedure on serum cortisol concentration**

Body weight of the piglets ($P < .001$) was retained in the final multivariable model for serum cortisol concentration. For consistency, weekly mortality rate was retained in the multivariate model even though it was not statistically significant ($P = .85$). The conclusions did not change regardless of whether mortality rate was or was not retained in the model.

Serum cortisol concentrations were highest 1 hour after castration or sham castration for piglets in the CF, SF, and C groups and 4 hours after sham castration for piglets in the S group (Figure 2). Maximum serum cortisol concentrations were 54.2, 34.1, 72.8, and 34.5 ng/mL for the CF, SF, C, and S groups, respectively.

At baseline, the serum cortisol concentration did not differ significantly ($P > .83$) among treatment groups (Figure 2). However, 1 hour after the procedure, piglets that underwent castration had significantly higher serum cortisol concentrations than did piglets that underwent sham castration (ie, group C vs group S and group CF vs group SF; $P \leq .02$). Four hours after the procedure, piglets in the CF group had significantly ($P = .03$) higher serum cortisol concentrations than did piglets in the SF group, but cortisol concentration did not differ significantly ($P = .32$) between piglets in the C and S groups. At 25 hours after the procedure, serum cortisol concentration did not differ significantly ($P > .21$) among treatment groups.

Compared with baseline concentrations, mean serum cortisol concentration was 61% higher for piglets in the CF group 1 hour after castration (Figure 3). In contrast, for piglets in the C group,
mean serum cortisol concentration was 96% higher 1 hour after castration, compared with the baseline concentration.

Discussion

Transdermal flunixin application is an attractive option to swine producers, given that it is a needleless method that can be applied prior to castration. However, results of the present study suggested that transdermal flunixin administration at a dose of 3.33 mg/kg was not effective in decreasing serum PGE₂ and cortisol concentrations in piglets undergoing castration. Further studies are needed to explore dosing regimens, including effective doses and administration frequencies, and the pharmacokinetics of flunixin following transdermal administration in piglets undergoing castration.

In the present study, serum PGE₂ concentrations did not differ between castrated piglets that received transdermal flunixin and those that received saline solution. These results contrast with findings of Bates et al., who demonstrated that meloxicam administered to the sow was effective at decreasing PGE₂ concentrations in piglets for up to 90 hours after drug administration. However, results from Bates et al. may not be directly comparable, given that the NSAID evaluated and the administration route were different from those in the present study. Unlike flunixin, meloxicam is considered a cyclooxygenase 2-selective inhibitor, and Bates et al. administered the drug to the piglets via the transmammary route of the sow. Given this administration route, sows were given a substantially higher dose than label recommendations (30 mg/kg) for 3 consecutive days. Therefore, these studies cannot be directly compared, and the lack of significant differences in our study may have been influenced by drug type, concentration, and administration frequency.

On the other hand, results of the present study are similar to those reported by Viscardi et al. Although not specific to pharmaceutical drug efficacy, Viscardi et al. measured PGE₂ metabolites to quantify differences in inflammatory response between 2 surgical castration methods (CO₂ surgical laser or scalpel vs sexually intact male control group). That study found no difference in PGE₂ metabolites between groups, and the authors suggested that the lack of differences may have been associated with the role of cortisol inhibition on the PGE₂ pathway. The synthesis of glucocorticoids (eg, cortisol) is a classic endocrine response to stress, and glucocorticoid production is part of an orchestrated biological mechanism that promotes gluconeogenesis, amino acid mobilization, and stimulation of fat breakdown to maintain the fight-or-flight response. Prostaglandin is a byproduct produced by this stress cascade, and cortisol concentrations can interrupt PGE₂ production.

This concept fits with the hypothesis postulated by Viscardi et al. and may have influenced the results of our study. In the current study, piglets administered flunixin prior to sham castration had the lowest PGE₂ concentrations 1 hour after the procedure, compared with all other treatments. This suggests that transdermal flunixin has the properties to mitigate PGE₂ concentrations, but perhaps only in situations when stress is controlled. Therefore, castration may induce enough of a stress response that NSAIDs cannot effectively mitigate inflammation unless given at a higher dose or greater frequency, such as in the study by Bates et al. Future work evaluating transdermal flunixin should use a higher drug dose or consider multiple administration to achieve concentrations great enough to compensate for the stress response.

An additional factor that may have influenced the lack of differences in PGE₂ concentrations between treatment groups in this study is sampling site collection. Blood samples in this study were collected via orbital bleeding, and physiologic changes to the orbital sinus represent physiologic changes occurring on a systemic level. Previous work by Thiry et al. demonstrated that transdermal flunixin was effective in reducing PGE₂ concentrations in bovine exudate following induced inflammation. However, that study used chambers to induce the local inflammation, and samples were collected directly from those chambers. Given that flunixin has a high degree of protein binding, distributes effectively throughout inflammatory exudate, and has a slow clearance rate, drug concentrations at the site of inflammation may exceed concentrations found in plasma. Therefore, in our study, PGE₂ concentrations may have been lower at the site of injury than in serum, and future work including interstitial probes as described by Nixon et al. may allow for a more accurate assessment of inflammatory mediation via PGE₂ in castrated piglets.

Analysis of cortisol concentrations in the present study demonstrated that surgical castration increased systemic cortisol concentrations, compared with sham concentration, and showed that cortisol remains an effective biomarker in assessing stress responses. Transdermal flunixin administration did not affect cortisol concentrations when comparing piglets undergoing castration. However, 1 hour after castration, the percent increase in cortisol concentration, compared with the baseline concentration, was lower for castrated piglets that received flunixin (61%) than for castrated piglets that received physiologic saline solution (96%). Coetzee et al. previously reported that male piglets nursing sows that received 2.0 mg of firocoxib/kg had a significantly (P = .04) lower mean serum cortisol concentration at 1 ± 1 hour after processing, compared with piglets nursing sows that received firocoxib at doses of 1.0 mg/kg or 0.5 mg/kg. The dose-dependent reduction in peak cortisol concentration after NSAID administration at the time of castration reported previously supports the assessment of cortisol as a surrogate marker of pain in piglets.

To the author’s knowledge, this was the first study to evaluate transdermal flunixin administration in piglets. Previous work in other species demonstrates mixed results in the efficacy of transdermal

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flunixin for mitigating pain and inflammation. A recent study evaluating transdermal flunixin administration in castrated bucklings demonstrated no effect on PGE2 or cortisol concentrations, whereas another study showed that flunixin-treated calves undergoing sham dehorning had higher PGE2 concentrations than did flunixin-treated calves that were surgically dehorned. Given that this was the first study to evaluate transdermal administration of flunixin in piglets and the drug dose was chosen on the basis of label recommendations for cattle, an appropriate and effective dose for piglet use has yet to be determined. Future studies should consider applying transdermal flunixin at various time points and potentially reapplying the drug to maximize concentrations. Given the unique qualities of transdermal flunixin, further research to identify an appropriate dosing regimen is critical given that with transdermal administration, piglets need to be handled only minimally and additional needle injections are not required.

Finally, given that flunixin is not label approved for use in swine, any use of flunixin in swine must be performed in compliance with the Animal Medicinal Drug Use Clarification Act.

Acknowledgments

This research was funded by a Veterinary Pharmacology Research Foundation Grant.

The authors declare that there were no conflicts of interest.

The authors thank Dr. Mary Battrell, Mark Underwood, Marco Lopez, and farm staff at farm S2 for allowing access to farms and support during data collection. In addition, the authors thank Matthew Browning and Jasmine Olivares for assisting with data organization and blood sample preparation.

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